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The development of the mammalian outer and middle ear

Neal Anthwal and Hannah Thompson

Craniofacial Development and Stem Cell Biology, King's College London, London, UK

Abstract

The mammalian ear is a complex structure divided into three main parts, the outer, middle and inner ear. These parts are formed from all three germ layers and neural crest cells, which have to integrate successfully in order to form a fully functioning organ of hearing. Any defect in development of the outer and middle ear leads to conductive hearing loss, while defects in the inner ear can lead to sensorineural hearing loss. This review focuses on the development of the parts of the ear involved with sound transduction into the inner ear, and the parts largely ignored in the world of hearing research: the outer and middle ear. To highlight the differences, in the last two years there were 8 papers and 11 papers published on the outer and middle ear respectively, while there were 22 inner ear papers published in the last 3 months alone. The published data on the embryonic origin, signalling, genetic control, development and timing of the mammalian middle and outer ear are reviewed here along with new data showing the Eustachian tube cartilage is of dual embryonic origin. The embryonic origin of some of these structures has only recently been uncovered (Thompson and Tucker, 13; Minoux et al, 13), while the molecular mechanisms controlling the growth, structure and integration of many outer and middle ear components are hardly known. The genetic analysis of outer and middle ear development is rather limited, with a small number of genes often affecting either more than one part of the ear or having only very small effects on development. This review therefore highlights the necessity for further research into the development of outer and middle ear structures, which will be important for the understanding and treatment of conductive hearing loss.

Keywords: Middle ear, Outer ear, Embryonic origin, Development, Ossicles, External auditory meatus, Eustachian tube cartilage, Tympanic membrane

Introduction

The mammalian ear can be divided into three main parts; the outer ear, middle ear and inner ear, all of which are required for effective hearing (Figure 1). The outer ear comprises the pinna, which is visible on either side of the head; its function is to funnel sound waves into the ear canal towards the tympanic membrane (eardrum). The middle ear is an air-filled

cavity found within the temporal bone of the skull. It is lined with a mucosa continuous with the Eustachian tube and houses the three middle ear ossicles that are suspended within it. The malleus (hammer), incus (anvil) and stapes (stirrup) form a chain between the tympanic membrane and the oval window that transmits sound waves from the outer to inner ear. The footplate of the stapes connects to a membrane stretched across the oval window; this vibrates with ossicular movement passing the sound waves to the inner ear. The inner ear is involved with balance as well as hearing. Within the cochlea of the inner ear the sound waves are converted into electrochemical signals to be passed to the brain. In order for the ear to function correctly all parts of the ear must develop in parallel in a strictly coordinated fashion. If any of these parts fail to form precisely then hearing will be impaired. In this review, we will focus on the development of the outer and middle parts of the organ of hearing.

The ear is a composite structure derived from tissues of neural crest, mesoderm, endoderm and ectodermal origin, and includes the bones of the auditory bulla and ossicles, the cartilage of the outer ear and Eustachian tube, muscles, nerves, blood vessels and epithelial membranes performing various functions. The lining of the ear canal is continuous with the skin and the outer surface of the tympanic membrane both of which are ectodermally derived; whereas the lining of the middle ear cavity is of dual origin (Thompson & Tucker, 2013), and is continuous with the Eustachian tube and the inner surface of the tympanic membrane. The ossicles and the tympanic ring are primarily of neural crest origin (see below), while the middle ear muscles are of mesodermal origin.

Embryonic origin of the ear tissues

In order to understand the development of the ear, we will first explain the development of the pharyngeal apparatus, a transient embryonic structure that develops into the lower face and neck. In all vertebrates, including mammals, the anterior of the developing embryo becomes segmented, with divisions along the rostral caudal axis. These are known as the pharyngeal arches. The first arch is the most rostral; it divides into the maxilla and mandibular arch and will form the upper and lower jaws (Figure 2). The second (hyoid arch) and more caudal arches will form the structures of the neck. The ear is composed of tissues from the first and second pharyngeal arches (Novacek, 1993; Moore & Persaud, 1993; Kontges & Lumsden, 1996; O’Gorman et al., 2005). The arches are lined externally by ectoderm and internally by endoderm; these have pockets of ectodermal clefts and

endodermal pouches that meet to divide the mesenchyme into the individual arches. The first pharyngeal pouch extends towards the ectoderm in the vicinity of the developing otic vesicle to form the tubotympanic recess that later becomes the Eustachian tube and the lining of part of the middle ear cavity. The arches are filled with neural crest cells surrounding a mesodermal core (Figure 3B). The neural crest-derived mesenchyme gives rise to skeletal elements of the jaw, connective tissue, tendons, pericytes and smooth muscle cells of the arch arteries, while the arch arteries and most skeletal muscles derive from the mesodermal cores (Noden, 1983; Noden, 1988; Trainor et al., 1994). The neural crest cells fill the developing pharyngeal arches as streams migrating from the dorsal neural tube. The first arch is filled with neural crest from the midbrain and the first and second rhombomeres, while the second arch is filled with neural crest cells predominately from the 4th rhombomere (Lumsden et al., 1991; Serbedziji et al., 1992).

The development of the pinna, ear canal and tympanic membrane

Capture and initial transmission of sound is facilitated by the pinnae and ear canal, which together form the outer ear. Compared to the inner and middle ears little is known about the development of the outer ear. Proper development of the outer ear is essential for functional hearing. It is therefore important to understand the development of this composite structure, as defects of the outer ear lead to conductive deafness in a number of syndromes and non-syndromic conditions. The current knowledge, such as it is, will now be reviewed.

The pinna, or auricle, is first seen as six small mounds, called the six hillocks of His at E11.5 in the mouse and 6 weeks gestation in humans (Table 1). These hillocks grow and eventually fuse to form a distinctive cartilage-filled structure surrounding the entrance to the ear canal. While in humans it is largely reported that the hillocks of His form at the border between the 1st and 2nd pharyngeal arches (reviewed in Carlson, 2014; Schoenwolf et al., 2012; Snow & Wackym, 2009) there is still controversy regarding the developmental origin of each of the auricular hillocks and which part of the mature pinna they will form. Evidence from human developmental disorders suggests the tragus is derived from the first otic hillock of the first arch, while the remaining hillocks are of second arch origin (Sauter et al., 2006). It has been recently shown by Minoux and coworkers (2013) using genetic lineage tracing experiments, that in mouse the pinna is derived of second arch neural crest and does not form at, but is near the arch border. In these experiments the 2nd pharyngeal arch neural crest cells were labelled using a *rhombomere (R)4::Cre* mouse line (Oury et al., 2006) crossed with the *Z/AP* (Lobe et

al., 1999) or *Rosa-CAG-LSL-tdTomato (Ail4)* (Madisen et al., 2010) reporter line. This revealed that the whole mouse pinna was derived from rhombomere 4, second arch neural crest cells and not a mixture of first and second arch crest as previously proposed (Minoux et al., 2013). As stated above, the consensus in humans is a dual origin pinna, suggesting there is not evolutionary conservation of the origin of the whole pinna in all mammals.

Once the hillocks of His have fused, the pinna continues to develop peeling away from the head at P3 in mice and 18 Gestational weeks (gw) in humans and reaches its adult morphology at P7 and 22gw respectively (Table 1). At this stage an elastic cartilage is present to support the shape of the pinna while allowing bending and movement. In many mammals the pinna is able to move independently of the head to optimise spatial hearing. Although the pinna maintains its morphology and completes most of its growth by 9 years of age (in humans), it continues growing slightly throughout life to become larger in males compared to females (Sforza et al., 2009).

Loss of function mutations in mice and genetic analysis of humans with non-syndromic microtia (Brown et al., 2013) have begun to reveal the genes involved with external ear development. Microtia, is a condition in which the pinna doesn't develop correctly to varying degrees, and is more commonly unilateral (79-93%) than bilateral, effecting the right ear more often than the left (~60%) and occurs in males more than females (1.5:1)(Alasti & van Camp, 2009). One of the genes known to have an important role in pinna development is the homeobox transcription factor *Hoxa-2*, which is essential for patterning the anterior-posterior axis in vertebrates and its loss in mouse pharyngeal arch 2 leads to a homeotic transformation into first arch tissues (Gendron-Maguire et al., 1993; Rijli et al., 1993). Additionally partial loss of human *HOXA2* function results in bilateral microtia with abnormally shaped pinnae (Alasti et al., 2008), demonstrating the conserved role of *Hoxa-2* in outer ear specification. This gene is important for various steps in pinna development. When *Hoxa-2* is lost in mice before E11.5 no pinna develops, however if it is lost between E12.5 and E13.5 then a hypomorphic pinna forms (Santagati et al., 2005). Furthermore, ectopic *Hoxa-2* in first arch neural crest cells is sufficient to induce a duplication of the pinna (Minoux et al., 2013). These data indicate that *Hoxa-2* is responsible in orchestrating the pinna morphogenetic programme. *Hoxa-2* regulates the expression of the transcription factor *Eya1* (Minoux et al., 2013), the gene implicated in Branchio-Oto-Renal syndrome in humans (BOR; OMIM: 113650; Abdelhak et al., 1997). In the absence of *Eya1* in mice the pinna is either absent or malformed (Xu et al., 1999), showing the essential role

of this gene for normal development. It is not yet clear how these transcription factors direct pinna development, however, *Hoxa-2* organises patterns of cell proliferation in the mouse, which will contribute to the control of pinna size and shape (Minoux et al., 2013). Minoux and co-workers (2013) also revealed a functional and genetic link between *Hoxa-2* and the downstream targets- *Bmp4* and *Bmp5*, both of which result in external ear phenotypes when expression is lost in mice (King et al., 1994).

A number of other genes have a demonstrable role in pinna development and will now be briefly reviewed. The *Fgfr1* “Hushpuppy” mutant mice have small, low set and pointed pinna (Pau et al., 2005; Calvert et al., 2011). Homozygous *Endothelin1* mutants have hypoplastic outer ears (Kurihara et al., 1994). The *Dumbo* mice have a mutation in the gene *Hmx1* (*Nkx5-3*), and have large, protruding, low set ears (Munroe et al., 2009) while humans with mutations in *HMX1* have pinna deformities (Schorderet et al., 2008). Treacher-Collins syndrome, caused by mutations in *TCOF1*, is associated with a number of craniofacial perturbations including pinna deformities and atresia of the external ear canal (Dixon, 1995). *Tbx1* mutant mice fail to form pinna when deleted either globally or conditionally in the 1st pharyngeal pouch (Jerome & Papaioannou, 2001; Arnold et al., 2006). The mouse knockout for *Prx1* (previously known as *MHox*) results in hypoplastic low set external ears (Martin et al., 1995), while the double knock out for *Prx1* and *Prx2* has a range ear defects not seen in the single mutants, including an absence of pinnae (ten Berge et al., 1998). Exactly how these genes function, what their targets are, and how they might interact in the coordinated control of pinna development is awaiting further study.

The ear canal has long been proposed to develop from the first pharyngeal cleft at the border between the first and second arches (as reviewed in Schoenwolf et al., 2012; Carlson, 2014; Mallo & Gridley, 1996). While this may still be so in the majority of amniotes, a recent publication shows that in the mouse this is not the case (Minoux et al., 2013). The first pharyngeal cleft extends towards and makes contact with the first pharyngeal pouch; it was thought that the pharyngeal cleft would then become the external auditory meatus and later the ear canal. However, even though the first pharyngeal cleft does extend towards and make contact with the first pharyngeal pouch in the mouse, this contact is not maintained and a new structure surrounded completely by first arch tissue invaginates from the first pharyngeal arch ectoderm to form the future ear canal. Minoux and co-workers (2013) analysed E14.5 *Wnt1::Cre;Hoxa2^{EGFP(lox-neo-lox)/+}* mice extensively, where expression of GFP is seen in second arch tissue, and showed the external auditory canal is surrounded completely by

Hoxa2/EGF-negative neural crest, and is therefore not at the border between the first and second arches.

The development of the external auditory meatus (EAM) starts as an invagination of the ectoderm extending towards the developing middle ear structures (Table 1; Figure 3). A meatal plug (or meatal plate; Figure 3; Table 1) forms at the end of the invagination. Later this meatal plug opens to form the ear canal and surface of the tympanic membrane, at the same time cornification of the ectoderm-derived stratified squamous epithelium occurs. Keratin squames can be seen in the forming cavity during this opening process (Michaels & Soucek, 1989; Nishimura & Kumoi, 1992; Nishizaki et al., 1998; Schoenwolf et al., 2012; Figure 3). Maturation and cornification of the epithelium of the ear canal occurs at the same time as in the skin and therefore is unlikely to be controlled by a local signalling event. Programmed cell death in the epithelium of the EAM may be important for the development of the meatal plug, as seen by TUNEL analysis (Nishizaki et al., 1998). However extensive TUNEL-positive cell death is not seen during the opening of the ear canal (Nishizaki et al., 1998) suggesting this developmental step could involve the initiation of cornification in this epithelium: as cornification involves a non- apoptotic cell death mechanism which is TUNEL- negative (Candi et al., 2009).

The extension of the EAM towards the developing middle ear is likely to be via a directed growth towards the tympanic ring, with a possible guidance cue being secreted from this region. Evidence for this can be seen in the absence of the tympanic ring in the *gooseoid* and *Prx (MHox)* mutant mice where the EAM does not extend (Rivera-Perez et al., 1995; Yamada et al., 1995; Mallo et al., 2000), in mice treated with retinoic acid, where the EAM is absent (Mallo & Gridley, 1996) and in human patients with *Gooseoid* mutations that have ear canal atresia (Parry et al., 2013). Additionally, in the *Hoxa-2* mutant mice where duplication of the tympanic ring occurs, there is also a duplication of the EAM (Gendron-Maguire et al., 1993; Rijli et al., 1993; Mallo & Gridley, 1996). Both of these EAMs have directed growth towards the two independent tympanic ring primordia (Mallo & Gridley, 1996), further supporting a guidance cue. The responsible guidance signal emanating from the tympanic ring, acting on the EAM, remains unknown. For the eardrum to form correctly the inner and outer epithelial surfaces need to be guided towards each other to reach their final position. These two epithelia develop independently controlled by two different mechanisms. The 1st pharyngeal pouch appears to form correctly in the absence of the EAM in the *gooseoid* mutant mouse (Rivera-Perez et al., 1995). Equally in the absence of the 1st

pharyngeal pouch the EAM is able to develop normally (Mallo & Gridley, 1996; Mallo, 1997). The mechanisms and molecules involved with this guidance process remain elusive, however the above studies indicate targets of *gooseoid* transcription would be likely candidates. Another gene important for EAM development is the transcription factor *Foxi3*; in *FOXI3*^{+/-} dogs and the single reported human *foxi3* ^{+/-} patient, the ear canal is completely absent and the outer ear is hypoplastic (Tassano et al., 2015). However, the middle ear appears normal. This highlights an important role for Foxi3 in EAM development, although whether it is involved with initiation, extension or opening of the EAM remains unclear. Unfortunately the current *Foxi3* heterozygous mouse has no phenotype (Edlund et al., 2014), and therefore cannot be used to elucidate the developmental mechanism controlled by this transcription factor, while the homozygous mutant has a severe disruption in the middle and external ear.

The ear canal is lined by modified apocrine sebaceous glands called ceruminous glands, and by sebaceous glands, both of which are associated with hair follicles (Perry & Shelly, 1955; Niemann & Horsley, 2012). These glands appear at P3 in the mouse and are fully differentiated by P10 (Gruneberg, 1971). In humans they are first seen at 13gw (gestational weeks) and are fully mature by 6 gm (gestational months), however they do not reach full functional capacity until puberty, at the same time as the final differentiation of the skin sebaceous glands (reviewed by Wright, 1997), suggesting a common developmental driver. These glands produce a viscous secretion and sebum that mixes with keratin squames to form earwax. In addition to these numerous small glands, the mouse has a single large earwax gland, the glandula ceruminosa, first seen at E15; this gland has a large duct that opens into the EAM not far from the eardrum (Gruneburg, 1971; Figure 4). The morphogenesis and molecular development of these specialised sebaceous glands hasn't been specifically studied, however they are likely to follow the same epithelial-mesenchymal signalling mechanism as other ectodermal glands (reviewed by Niemann & Horsley, 2012). In skin sebaceous gland development, altering hedgehog- or Wnt- signalling affects the formation of sebaceous glands (Allen et al., 2003; Niemann et al., 2003) suggesting a key role for these pathways in sebocyte cell fate. Whether these genes also have a key role in ceruminous gland development in the ear canal could be confirmed by altering Hedgehog- or Wnt-signalling pathways in culture of external auditory meatus epidermis or by looking *in vivo* at transgenic mice where these pathways are altered. However, it could be that these

modified ceruminous glands follow a unique developmental pathway allowing the production of earwax.

The tympanic membrane (commonly called the eardrum) has an outer layer formed of and continuous with the ectoderm of the ear canal, and an inner layer said to be of endoderm, (reviewed by Schoenwolf et al., 2012; Cochard, 2012; Carlson, 2014), however this has never been shown via embryonic tracing methods. The ectodermal outer layer is stratified squamous epithelium, which shows a unique lateral migration of epidermal cells from the centre of the eardrum to the edges where they then can desquamate (Alberti, 1964; Litton, 1963; Boedts & Kuijpers, 1978). This process accounts for the self-cleaning ability of the outer ear. The inner layer is a simple squamous epithelium (Lim, 1968a; Lim, 1968b). The mammalian eardrum can be divided into two regions based on morphology, the ventral pars tensa, which as its name suggests is a tense structure suitable for vibration; and the dorsal pars flaccida, which is more elastic (Shrapnell, 1832). The pars flaccida (Shrapnell membrane) is the upper region of the eardrum above the malleolar folds and is relatively fragile compared to the larger part of the eardrum, the pars tensa (Figure 5). The pars flaccida is often mis-reported to be composed of only two layers of epithelium with a small number of elastic fibres between them (Snow & Wachym, 2009; Probst et al., 2005), however an in-depth study using electron microscopy distinctly shows three layers: an outer epidermal layer, a middle layer of loose connective tissue and an inner simple mucosa (Lim, 1968b). This tri-layered structure therefore is similar to the pars tensa, and both can be described as having an inner layer of neural crest cells in the form of a loose connective tissue, sandwiched between two epithelial sheets (Lim, 1968a; Lim, 1968b). This inner layer is the lamina propria that in the pars tensa consists of two layers of thin fibrous collagen-rich connective tissue: an outer layer of radially directed fibres (radiate layer) and an inner layer of circular fibres (circular layer) (Lim, 1968a; Probst et al., 2005); while in the pars flaccida no regular arrangement of extracellular matrix is seen (Lim, 1968b). The pars flaccida varies in size and shape between mammals, for example in the sheep it is elliptical and almost the same size as the pars tensa, while it can be regressed as in guinea pigs (Chole & Kodama, 1989; Lim, 1968b; Shrapnell, 1832). The pars flaccida therefore, is different to the pars tensa at a gross and cellular level (Shrapnell, 1832; Lim, 1968a; 1968b). The structural and functional differences between these two regions may explain why retraction pockets of the tympanic membrane occur more commonly in the flexible pars flaccida than the inflexible pars tensa (Maw et al., 2011). Further structural changes are seen during postnatal human development, with the tympanic

membrane changing from a horizontal to vertical orientation by the time of maturity (Ikui et al., 1997). This change in orientation likely occurs as a consequence of the postnatal growth of the lower face, which occurs throughout adolescence.

After being neglected for much of the modern developmental biology era in recent years research had begun to investigate the development of the outer ear using modern molecular techniques such as using transgenic mice. Despite this there are still many unanswered questions relating to the finer detail of the development and morphogenesis of the outer ear. This includes answering how morphogenesis of the ear canal occurs in light of the fact that it does not form from the 1st pharyngeal cleft, and whether this process of ear canal development is common throughout mammals, and even across other tetrapods. Mutations causing defects in the outer ear, such as *Tcof1*^{-/-}, *Hmx1*^{-/-} and *Tbx1*^{-/-} also affect the middle ear, suggesting a tight integration and coordination of the two parts. Whilst knowledge of the development of middle ear is poor when compared to the inner ear, it is a great deal better understood than the outer ear. This knowledge on how the middle ear develops, and the embryonic origins of its various components will now be addressed.

The development of the middle ear skeletal structures.

The three mammalian middle ear ossicles are formed by a process of endochondral ossification from neural crest of the first and second arches (Figure 2C). The malleus and incus are predominately formed from first arch crest, with second arch crest only contributing to the orbicular apophysis of the mouse malleus (O’Gorman, 2005). As Mason highlights, the mouse orbicular apophysis is often mistaken for the processus brevis, including in the above study by O’Gorman (Mason, 2012). Due to this misidentification it remains unknown whether the processus brevis is a first or second arch derivative, and whether the malleus of species without an orbicular apophysis has a dual arch origin. Furthermore, as this group of mammals without an orbicular apophysis includes humans (Mason, 2012) inaccurate descriptions may have consequences in the clinical application of mouse derived data. By way of example the *Msx1* mutant mouse is lacking the orbicular apophysis, but is described in the original study as having a missing processus brevis (Zhang et al. 2003).

The malleus and incus form from Meckel’s cartilage, a continuous rod of cartilage that extends throughout the lower jaw and ends in the incus. The caudal end of this structure later subdivides to separate these two ossicles (Amin & Tucker, 2006; Figure 6). This process is controlled not by apoptosis as in other joints, but by a down regulation of cartilage markers

such as type 2 collagen and the switching on of joint markers such as Tgf-beta super family member *Gdf5* (Amin et al., 2007; Figure 6C). Most members of the sub-order of rodents Ctenohystica possess a fused malleus and incus that acts as a single morphological and functional unit (Mason, 2012). Guinea pigs, one such member of this sub-order, follow the same pattern of development as those mammals with a functional malleo-incudal joint, with separation of the ossicles by down regulation of cartilage markers and upregulation of joint markers in the 'joint' region. However, this joint then collapses in late development and these ossicles are fused with a suture demarking the two elements (Amin & Tucker, 2006).

The manubrium of the malleus is inserted into the tympanic membrane in order to transmit vibrations of the eardrum into the ossicular chain. The close physical and functional association of the manubrium and the tympanic membrane suggests tight control of their integration is essential for normal function. Using *in vitro* techniques and transgenic or retinoic acid (RA)-treated mice lacking the EAM, Mallo and co-workers (1996; 2000), have shown the EAM signals to induce chondrogenic differentiation of the manubrium, and in its absence the manubrium fails to form. Ossification of the ossicles begins with the incus at 16 gestational weeks in humans, followed by the malleus between 16-17 weeks and the stapes at 18 weeks and is finished around 26 weeks (Scheuer & Black, 2000). The malleus and incus initially form a bone marrow cavity, possibly involved with blood formation in early infancy; this gradually disappears in the first 2 years of postnatal life (Yokoyama et al., 1999). Therefore, the malleus and incus could be said to continue developing for much longer than simply the pre- and neo-natal period.

During much of embryonic development, the malleus remains attached to the mandible via Meckel's cartilage. In mice separation of the malleus and the mandible occurs from postnatal day 1 (P1) by the onset of the breakdown of the neighbouring Meckel's cartilage. The separation process is complete by P3, and the remaining Meckel's cartilage continues to breakdown as part of its presumed transformation into the malleomandibular ligament, the proximal portion of which is the anterior ligament of the malleus (Anthwal et al., 2013; Stevens-Sparks & Strain, 2014; Figure 6). The timing of this separation suggests that changes in the mechanical stress acting upon the jaw due to the onset of feeding may have an influence on the breakdown of this cartilage. This is further supported by the timing of breakdown seen in marsupials, where Meckel's cartilage remains attached to the malleo-incudal joint, which forms the early jaw joint in early postnatal stage, until the development of the tempromandibular joint (Clark & Smith, 1993; Sánchez-Villagra et al., 2002). The

signalling events governing the breakdown and transformation of Meckel's cartilage are poorly understood. The *Fgf* and *Tgf-beta* signalling pathways may have a role in the prevention of differentiation of this cartilage into bone (Ishizeki et al., 2010; Oka et al., 2007). The breakdown of Meckel's cartilage appears to be driven by an apoptosis-independent mechanism (Trichilis & Wroblewski, 1997; Harada & Ishizeki, 1998). In the absence of programmed cell death a series of studies has begun to shed light onto the role of matrix metalloproteinases in the breakdown of the distal cartilage in mice (Sakakura et al., 2005; Sakakura et al., 2007; Sakakura et al., 2008; Sakakura, 2010). This region of Meckel's cartilage breaks down at E15.5 in the mouse, 6-7 days before the breakdown of the proximal cartilage, and appears to disappear in order to make room for the growing dentition. As the proximal cartilage breaks down in a different developmental milieu, with the distal cartilage being replaced by the dentary bone and the proximal transforming into ligament, it is likely that different signals are directing these two processes.

The stapes is the mammalian equivalent of the single non-mammalian terrestrial vertebrates' columella, derived from the rostral end of Reichert's cartilage (Moore & Persaud, 1993). A stirrup-shaped structure with its head connected to the incus and its two arms surrounding the stapedial foramen to join the stapedial footplate, which is inserted in the oval window. The footplate of the stapes attaches to the otic capsule via the mesodermal annular ligament, which is a circular ligament surrounding the stapes. The head, arms and inner footplate of the stapes are composed of neural crest of second arch origin, while the outer parts of the stapedial footplate are of mesodermal origin (O'Gorman et al., 2005; Thompson et al., 2012; Figure 7). The origin of the stapedial footplate was questioned for over a hundred years, mostly based on studies performed in the chick using chick-quail grafting techniques (Le Lievre, 1978; Couly et al., 1993; Noden, 1986). However, recent work published by Thompson and colleagues (2012) using reporter mice lines showed conclusively that the footplate is of dual origin. In mice, the stapes appears to develop in close association with the vestibule of the inner ear, already inserted within the oval window of the otic capsule (Thompson et al., 2012). The neural crest-derived footplate is needed for the development of the mesodermal-part of the stapes footplate, as in the absence of neural crest cells no footplate is seen within the oval window. This suggests the development of this composite structure is highly regulated to coordinate accurate integration, in order to produce a functional unit of hearing. The development of the oval window itself appears to be linked to the development of the stapes; this is evident by looking at mutant mice lacking the stapes,

where the oval window is reduced to a small dimple (Thompson et al., 2012; Kanzler et al., 2000). However, the positioning of the reduced oval window remains the same in these mutants, suggesting the initiation of the oval window may be due to another local factor, such as the underlying otic epithelium.

There are a large number of human cases of stapes fixation, where the annular ligament is absent or has become ossified preventing movement of the stapes within the oval window and therefore causing hearing loss (Potti et al., 2011; Thys & Van Camp, 2009; Huang et al., 2005; Nandapalan & Tos, 2000). This commonly occurs during postnatal life, but also can be caused congenitally (Nandapalan & Tos, 2000). The positioning of the annular ligament as it encircles the stapes footplate is between the mesodermal part of the stapes footplate and the mesodermal otic capsule. The factors involved with the specification and development of the annular ligament have not been studied, however, the Bmp antagonist noggin, is expressed around the stapes footplate potentially inhibiting skeletogenesis in this region (Hwang & Wu, 2008). Additionally, human patients with Multiple Synostoses Syndrome 1 (OMIM: 186500) and Stapes ankylosis with broad thumbs and toes (OMIM: 184460) both have stapes fixation and have been linked to the NOGGIN locus (Potti et al., 2011).

The tympanic ring is derived from neural crest of the first pharyngeal arch and forms by intramembranous ossification serving to support the tympanic membrane (Novacek, 1993). It later becomes integrated into the auditory bulla of the temporal bone. The transcription factor *Gooseoid* (*Gsc*) is needed for the mesenchymal cells to condense into the tympanic ring structure and is later also needed for osteogenic differentiation of these cells (Rivera-Perez et al., 1999). The tympanic ring is anchored to the malleus via the anterior process (processus gracilis, folianus, or longus) of the malleus. This anterior process is derived from the gonial bone, which like many of its closely associated elements is derived from the first pharyngeal arch neural crest cells (Rijli et al., 1993). During early postnatal development in the mouse the gonial forms by intramembranous ossification inferior to Meckel's cartilage, later invading into the anterior aspect of the malleus (reviewed in Anthwal et al 2013). This leads to the mature malleus being formed from dual endochondral and intramembranous origins. Gonial development requires *Bapx1*, with loss of gene function resulting in loss of the gonial bone (Tucker et al., 2004), whilst loss of *Gsc* results in a hypoplastic gonial (Yamada et al., 1995). In both of these mutant mice, changes in the gonial are accompanied by changes to the neighbouring tympanic ring. There are yet to be described

any mutants that result in a specific loss of the gonial, which would indicate the genes involved with its development. However, the previously mentioned *Dumbo* mouse, a mutant for the homeobox transcription factor *Hmx1* (*Nkx5-3*) results in a specific hyperplasia of the gonial (Munroe et al., 2009), offering some insight into the details of the developmental programme of this bone. It is worth noting that not all mammals are in possession of a gonial and this includes humans.

The auditory bulla encases the middle ear and defines the cavity in which the ossicles are suspended, later becoming integrated into the temporal bone. The otic capsule, which acts as the medial wall of the middle ear as well as supporting the membranes of the inner ear, is mostly derived of mesoderm with the exception of a small part near the oval window that is neural crest derived (Couly et al., 1993; O’Gorman et al., 2005; Thompson et al., 2012). The otic capsule undergoes endochondral ossification, beginning in the mouse during embryonic development. The outer walls of the auditory bulla are formed by part of the intramembranous squamosal bone, and are neural crest derived (Richter et al., 2010). In the mouse the bulla is apparent from P6 and is complete at P9 (Richter et al. 2010). The development of the bulla is poorly understood, possibly due to its postnatal development and the fact that mutants with disrupted squamosal bones, such as the *Foxc1*^{-/-}, *Fgf8*^{-/-} and *Dlx* mutants, have extensive abnormalities and are non-viable (Depew et al., 2005, Inman et al., 2013). Richter and colleagues have been able to illuminate auditory bulla development by demonstrating that mice heterozygous for *Tcof1*, a Treacher-Collins syndrome (OMIM: 154500) mouse model, have reduced auditory bulla volume associated with reduced proliferation in the membranous bone (Richter et al., 2010). Similarly, the *Fgf23* knockout mice have smaller auditory bullae with reduced bone density (Lysaght et al., 2014). Further investigation into other knockdown mutants may help to understand the development of the auditory bulla, elucidating the genes and mechanisms that control the integration of the components of the auditory bulla, its positioning and final shape.

Middle ear cavity formation

The middle ear cavity is lined by a mucous membrane covered by an epithelium of dual embryonic origin (Thompson and Tucker; 2013). Its function is to act as an interface between the air and the underlying tissue, forming tight junctions between the epithelial cells and keeping the tissue moist by the production of mucus from goblet cells. The region of the mucosa close to the Eustachian tube is a pseudostratified epithelium rich in goblet cells and

ciliated cells that function together to secrete mucus, trapping debris and pathogens, before wafting them towards and along the Eustachian tube to keep the middle ear cavity clear (Nuutinen et al., 1983). Other regions of the middle ear mucosal epithelium, further from the Eustachian tube, have been shown to be free of ciliated cells and goblet cells and are formed of a simple epithelium (Buch & Jorgensen, 1964; Lim et al., 1967; Kuijpers et al., 1984; Nishizaki et al., 1997). These differing epithelial patterns are seen in human, guinea pig, rats and mice indicating commonality across mammals. The formation of the middle ear cavity starts with the invagination and extension of the first pharyngeal pouch towards the developing inner and middle ear structures, to become the tubotympanic recess (Figure 8A). The invagination then ruptures near the distal end and becomes filled with surrounding neural crest cells (Park & Lim, 1992; Thompson & Tucker, 2013; Figure 8B). This initial process occurs while the middle ear ossicles are in the early stages of development and have just started to undergo condensation within the surrounding neural crest-derived mesenchyme. As the ossicles undergo chondrogenesis the whole of the future middle ear cavity is completely filled with mesenchyme, with only a single layer of endodermal epithelium present on the inner surface of the developing tympanic membrane and along the ventral regions of the future middle ear cavity (Figure 8C). The neural crest cells that fill the cavity later regress, eventually freeing the ossicles to leave them suspended within an air-filled space. This clearance occurs first in the ventral cavity (hypotympanum and mesotympanum) with the neural crest cells appearing to peel away from the endoderm of the ventral tympanic membrane towards the otic capsule (Figure 8D & E). Next is the clearance of the attic region (epitympanic recess) in the vicinity of the ossicles, with the last neural crest cells clearing from the dorsal most region of the middle ear cavity above the ossicles (unpublished observations; Figure 8F). This process of cavitation has been shown in mice, however in order to be clinically relevant for humans it needs to be confirmed by looking at the development of this region in human embryonic tissues. As the structure of the mouse and human middle ears are remarkably similar it is likely that the process of middle ear cavitation seen in mice is relevant across the whole mammalian species.

Following cavitation the neural crest cells now lining the cavity in the attic and on the cochlea promontory undergo a mesenchyme-to-epithelium transformation to form an epithelium continuous with the endoderm-derived epithelium (Thompson & Tucker, 2013). This was shown using *Wnt1cre/R26R* (Danielian et al., 1998; Soriano, 1999) reporter mice lines, to label the neural crest cells, double labelled with epithelial markers to show neural

crest cells producing epithelial cell proteins. This process of cavitation occurs postnatally in mice and rats, but in humans it occurs *in utero* between the 3rd and 8th month of gestation; suggesting the postnatal activities such as feeding and breathing have no control over cavitation. In the *Tcof* mutant mice a smaller auditory bulla results in retained mesenchyme within the middle ear cavity, suggesting complete cavitation requires proper auditory bulla growth (Richter et al., 2010). Therefore the neural crest cells within the embryonic middle ear cavity are simply redistributed around the edges of the much larger postnatal auditory bulla rather than being removed from the cavity by processes such as programmed cell death. Further evidence to support this comes from work in the opossum and in humans where the volume of mesenchyme within the middle ear at various times was calculated and found to remain the same (Piza et al., 1998). Additionally, no extensive apoptosis has ever been detected within the middle ear mesenchyme that would explain the clearance (Roberts & Miller, 1998). The dual origin of the middle ear epithelium is unique to mammals and the development of the three-ossicle mammalian middle ear may have lead to its evolution (Thompson & Tucker, 2013).

The mastoid process of the temporal bone, homologous to the petrotic or petrosal bone, is found dorsal to the middle ear cavity and in humans is composed of a honeycomb of air-filled spaces. In humans, pneumatization of the mastoid air cells occurs mostly up to the age of 5, after which growth slows until its final size at around 10 years in girls and 15 years in boys (Rubensohn, 1963). As mastoid pneumatization occurs there is some degree of bone remodelling likely by osteoclasts and large numbers of apoptotic osteocytes are seen (Fujiyama et al., 2013). This bone remodelling is essential for the formation of the air cells within the mastoid process and the epithelial lining must be able to expand to fill this enlarging space. The role of the mastoid air space is poorly understood. A suggested role in humans is that of a regulator of pressure in the middle ear cavity (Doyle, 2007). Equivalent structures to the mastoid airspaces vary across species. The morphology ranges from animals with no airspaces, such as mice and rats, via cat-like carnivores with bony septae dividing the middle ear cavity into two compartments, to those animals with one or more air spaces of interconnected air cells (Rosowski, 2013). The mastoid air-cells and homologous air spaces are lined by an epithelium directly linked to the attic region of the middle ear and therefore can be supposed to cavitate in a similar way to the middle ear. At present this is only a hypothesis as the most commonly studied mammalian model organisms of development, mice and rats, do not possess mastoid air cells and therefore the development of this region is

not well understood. Guinea pigs possess an air-filled posterior recess of the temporal bone, and as such it offers an amenable model for the human mastoid. Such models are clinically important, since development of the mastoid has been shown to be impaired when otitis media is prevalent in young children (Chen et al., 2014). However, the powerful genetic and molecular tools available in mice, such as gene knockouts and genetic lineage tracing systems, are not widely available in other mammalian model organisms. This is a common problem known to those using non-canonical model organisms. As such we are currently unable to determine if the lining of the mastoid air cells are endoderm or neural crest in origin. However we might hypothesise that, like the attic region of the mouse, the lining of the air-cells is neural crest- derived.

Development of other middle ear structures

In addition to the skeletal structures of the middle ear, there are a number of other elements, which are present within the cavity and are needed for normal auditory function. These include the ossicular muscles, tendons and ligaments, the stapedia artery and the Eustachian tube. The middle ear muscles comprise the tensor tympani and stapedius, which function to dampen vibrations of the malleus and stapes in response to loud noises or the internal noises caused by mastication and speech, and act in part to protect the inner ear from damaging sound (Klockhoff & Anderson, 1960; Salomon & Starr, 1963; Borg, 1972; Brask, 1979). The tensor tympani inserts into the handle of the malleus and in reflex pulls this bone away from the tympanic membrane reducing vibrations of the eardrum and therefore sound volume. Innervation of the tensor tympani is from the tensor tympani nerve, a branch of the mandibular division of the trigeminal nerve (Kierner et al., 2002). The tensor tympani is derived from the mesodermal core of the first pharyngeal arch and is contiguous with the tensor veli palatini, extending from the malleus, out of the middle ear cavity and along the Eustachian tube (Schoenwolf et al. 2012; Kierner et al., 2002). The stapedius muscle forms from two anlagen; one for the tendon, which derives from the interhyale and the other for the main body of the muscle, which derives from the mesodermal core of the second pharyngeal arch (Rodriguez-Vazquez, 2009; Schoenwolf et al., 2012). The stapedius inserts into the neck of the stapes and is innervated by a branch of the facial nerve. The molecular control of the development of these specific muscles has not yet been analysed; the only detail we have, was found by looking at the *scleraxis* knockout mice. The tendons attaching the middle ear muscles to the ossicles express the transcription factor *Scleraxis* (*Scx*). In *Scx* null mice these tendons are shorter and malformed, which is likely to contribute to the hearing loss seen in

these mice (Wang et al., 2011). Within the middle ear there are 5 suspensory ligaments associated with the ossicles; these are the superior, anterior, and lateral malleal ligaments, and the superior and posterior incudal ligaments (Gelfand, 2011). The anterior ligament of the malleus is derived hypothesised to be of first branchial arch tissue and develop from Meckel's cartilage (Moore et al., 2015), although no lineage tracing has been performed. The other ligaments have not been described in such detail. The development of these ligaments is first seen as a condensation of mesenchyme in the 3rd gestational month in humans, collagen fibres are then laid down and differentiation is complete by the 9th month (Hartwein & Rauchfuss, 1987). These ligaments are critical for the suspensory positioning of the middle ear ossicles allowing proper conduction of sound waves through the middle ear (Huttenbrink, 1989), however at present no research has shed light upon the molecular development of these structures and so present an avenue for future studies.

The stapedial artery passes through the middle ear and between the two crus of the stapes in mammals. It forms from either the first and second pharyngeal arch arteries in mice (Hiruma et al., 2002), rat (Tandler, 1902), porcupine (Struthers, 1930) and hyrax (Lindahl & Lundberg, 1946), but only from the second arch artery in humans (Padgett, 1948). Interestingly the stapedial artery is only known to persist in adult mice, rats and hyrax, whereas it is a transient embryonic structure that later regresses in most individuals of all other species studied, including humans, rabbits and porcupine (Hiruma et al., 2003). However there is some variation in these species with a persistent stapedial artery being present in 0.5% of humans, and doesn't seem to affect the normal development of the remaining middle ear structures or impede hearing (Moreano et al., 2009).

The Eustachian tube is the connection between the middle ear and the nasopharynx. It is formed from the proximal end of the first pharyngeal pouch (tubotympanic recess), and is therefore lined by an endoderm-derived epithelium. This epithelium is a pseudostratified columnar epithelium containing goblet cells and ciliated cells, which function to trap and remove invading pathogens into the nasopharynx protecting the middle ear (Park et al., 1992). The skeletal elements of the Eustachian tube include a lateral bony part that arises from the anterior wall of the auditory bulla and a cartilaginous portion that covers the dorsal region along the length of the tube. The cartilage of the Eustachian tube is mostly of mesodermal origin, however a small part is neural crest derived (Figure 9). There are four muscles surrounding the Eustachian tube, with the first arch-derived tensor veli palatini and the fourth arch-derived levator veli palatini having the most crucial roles (Ishijima et al.,

2002). These muscles insert into the palate and function to open the Eustachian tube, from its normal closed form, during yawning, swallowing and mandibular movement (McDonald et al., 2012). If these muscles do not develop correctly, as in the *Tbx1*/+ and *Dfl*/+ (22q11.2 deletion syndrome) mice, then the Eustachian tube is unable to function to clear the middle ear cavity correctly resulting in otitis media (Fuchs et al., 2014). The Eustachian tube in adolescent humans is shorter, narrower and angled more horizontally (10°), as they age this angle changes to a more vertical position (45°) with a longer length allowing better drainage and reducing the occurrence of otitis media (Proctor, 1967; Ishijima et al., 2000). However, in a number of human syndromes, such as Down's syndrome (Mazzoni et al., 1994), Frankter Haar syndrome (Bendon et al., 2012) and Turner's syndrome (Sculerati et al., 1990) the Eustachian tube angle and position does not develop correctly due to a broad range of craniofacial defects: this again can lead to a higher incidence of otitis media.

Conclusions

The development of the mammalian middle ear involves a complex series of events tying in many tissues of different embryonic origins in order to produce a functional organ of hearing. As recently as 2013, work by Minoux and co-workers and Thompson & Tucker uncovered important details of the embryonic origin of parts of this organ. When compared to the inner ear, the development of the outer and middle ear is still poorly understood, with many of the most recent findings such as the origins of the ear canal and the dual origins of the stapes and middle ear epithelium yet to be translated to the human ear. The disparity in knowledge about the inner ear versus the outer and middle ears does not necessarily reflect a lesser prevalence of conductive hearing loss, due to defects in the outer and middle ears, compared to sensorineural hearing loss, which is due to problems in the inner ear. Instead, it may reflect that treatability of conductive hearing loss in the infant, with surgical and other solutions having been being available in many cases for some time (Briggs & Luxford, 1994; Baba et al., 2004), while sensorineural hearing is more permanent. However, since there are a large number of human cases where the development of the outer and middle ears is impaired often resulting in at least some degree of hearing loss, understanding their development is therefore important.

A few genes have been found to be important for the development of the outer and middle ears, including those mentioned above such as *Hmx1*, *Tcof1*, and *Eya1*, however they often affect more than one part of the ear and sometimes result in complete loss of the outer

and middle ear structures. Many of these genes, such as *Tcof1*, have been found in association with human craniofacial syndromes, which then need to be transferred to appropriate animal models before any detailed analysis can be performed on their function and significance. This means details of the genetics involved with ear development are still poorly understood and a lot of work is needed researching the fine detail and interactions of these genes and how they function specifically in outer and middle ear development.

Much of the information about outer and middle ear development has been gathered through the use of the laboratory mouse, and as such the universality of many developmental processes across mammals is yet to be confirmed. This is a common problem in developmental biology not confined to the study of the ear. Where technologically and ethically possible, conformational studies in other non-canonical model mammals should be carried out so as to understand the commonalities and variation of mammalian ear development.

It has been one intention of this review to clarify a number of descriptive errors and ambiguities pertaining to the middle and outer ear often seen in the literature. These include the orbicular apophysis commonly being misidentified as the processus brevis, and the pars flaccida being described as having only two layers of epithelium when in fact there is an intermediate layer of cells in between. We believe that these clarifications will help reduce confusion and prevent further descriptive mistakes in future studies of the middle and outer ears.

There are a number of important unanswered questions pertaining to the development of the outer and middle ear. These include, but are not restricted to: 1. Elucidating the formation of the outer ear canal, 2. Determining the development origin of the pinna across mammalian species, and 3. Discovering how the outer and middle ear development is coordinated resulting in the proper development of the eardrum. These questions demonstrate that, with regards to the development of the outer and middle ear, there is still a lot to do. As such it offers a fertile field of study with important clinical implications.

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Table 1. A table comparing the timings of development of different parts of the outer ear between mice and humans. Data from Michaels & Soucek, 1989; Nishimura & Kumoi, 1992; Miyake et al, 1996; Nishizaki et al, 1998; Mallo & Gridley, 1996 and unpublished observations. E- embryonic; P- postnatal. EAM- external auditory meatus.

	Mouse (Embryonic/ Postnatal days)	Humans (Gestational days/ weeks)
Hillocks of His seen	E11.5	6 gw
Hillocks fuse	E13	7-9 gw
Pinna detaches from head	P3	18 gw
Pinna achieves adult morphology	P7	22 gw
EAM open and extending	E9.5	55 gd
EAM closed	E15-P7	8 gw < 15 gw
EAM re-opening	P7-P12	13 gw < 21 gw
Ear canal open	P12	21 gw

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Figure Legends

Figure 1. Schematic illustrating the structure of the ear, which is divided into three parts, the outer, middle and inner ear. The outer ear includes the pinna and ear canal. The middle ear is between the tympanic membrane (eardrum; ty) and stapes (s), which is inserted in the oval window (ow). The middle ear is connected to the back of the mouth via the Eustachian tube. The inner ear converts the sound waves to electrical signals. M- malleus; I- incus; s- stapes;; Tt- tensor tympani; st- stapedius; EAM- external auditory meatus; ALM- anterior ligament of the malleus; SLM- superior ligament of the malleus; PLI- posterior ligament of the malleus; mn- manubrium; oa- orbicular apophysis.

Figure 2. Schematic illustrating the early setup of the ear. A) The pharyngeal arches are filled by neural crest streams; the first pharyngeal arch (PA1) is filled with neural crest cells from the midbrain (M), rhombomere 1 (r1) and rhombomere 2 (r2), while the second pharyngeal arch (PA2) is filled with crest predominantly from rhombomere 4 (r4). The ear is derived from the tissues of PA1 and PA2. B) The first and second pharyngeal arches are divided internally by the endodermal first pharyngeal pouch (1pp) and externally by the ectodermal first pharyngeal cleft (1pc). C) The middle ear is a composite structure derived from mixed embryonic origin, as seen in this schematic of an embryonic mouse middle ear. EAM, external auditory meatus; g, gonial; mm, manubrium of the malleus; Tr, tympanic ring; o, orbicular apophysis; M, malleus; I, incus; s, stapes; ow, oval window.

Figure 3. The development of the external auditory meatus (EAM) in mice. The development of the EAM in the mouse seen in frontal sections at E13.5 (top), E15.5 (middle) and P11 (bottom). The EAM (black arrow) extends towards the tubotympanic recess (white arrow) as an open structure at E13.5. By E15.5 this has collapsed to form a meatal plug (black arrow, and enlarged box) close to the tubotympanic recess (white arrow). At P11, the ear canal is opening and keratin squames can be seen within it (black arrow head). mec- middle ear cavity.

Figure 4. The glandula ceruminosa of the mouse. Trichrome stained frontal sections of P6 mouse outer ear from caudal to rostral, showing A) a lower power image for orientation; B) the ceruminosa gland duct (black arrow) where it joins to the EAM (white arrows), at its heel; C) the large duct of the ceruminosa gland (black arrow); D) and the gland itself (black outline). me- middle ear; oe- outer ear.

Figure 5. Ear drum development in the mouse. A) Schematic of the eardrum with pars flaccida and pars tensa. B) Schematic showing the pars flaccida (dorsal) and pars tensa (ventral) within the ear. Oe/ ec- outer ear/ ear canal; mec- middle ear cavity.

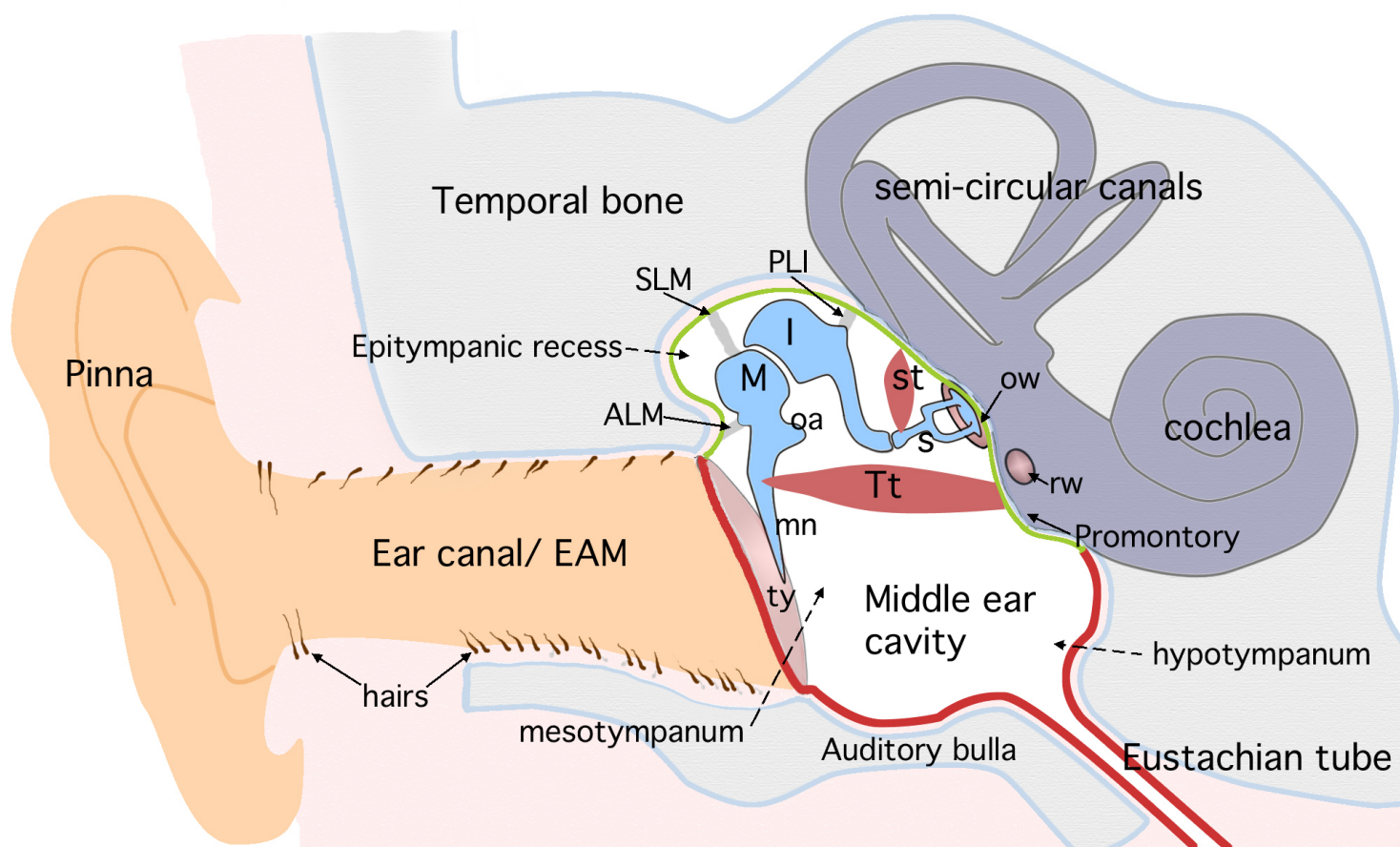
Figure 6. Development of the malleus and incus from Meckel's cartilage in the mouse. A, B- Alcian blue stained section of Meckel's cartilage at E13.5 (A) and E14.5 (B). C- Expression of the joint marker, Gdf5 on a sagittal section at E15.5, showing expression in the joint region between the malleus and incus. D, E- Alcian blue and Alizarin red stained skeletal preparations of the caudal end of Meckel's cartilage, showing the connection to the malleus

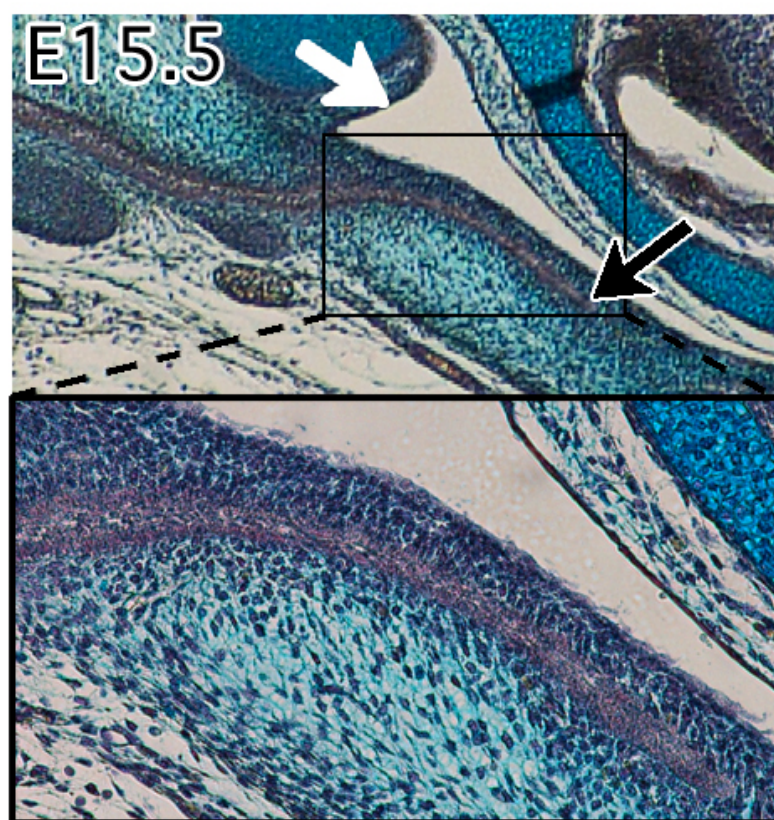
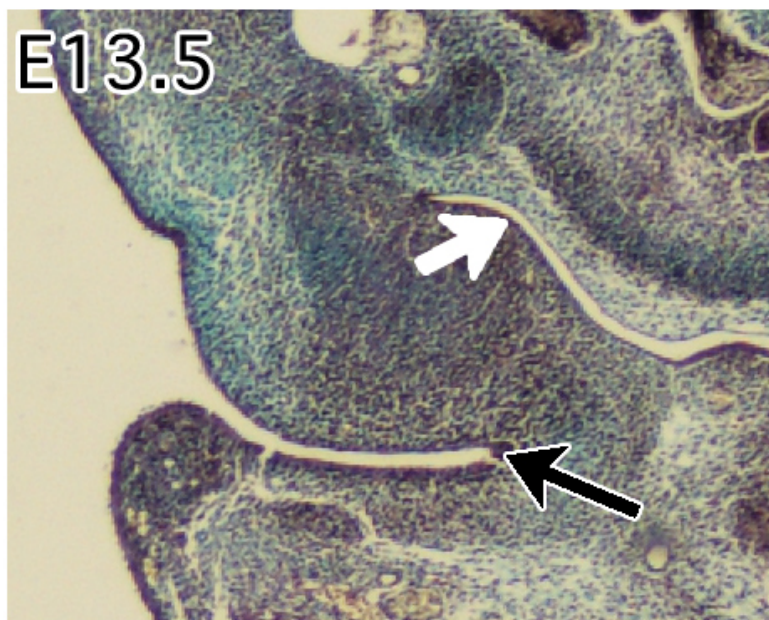
at P0 (D) and its separation at P3 (E). F- Schematic representing the separation of the ossicles, between E13.5 and P3. At E13.5 (A, F), the malleus and incus are still fused to the caudal end of Meckel's cartilage. At E14.5 (B, F), the malleus and incus have separated (arrow). (C) The joint marker, *Gdf5* is expressed in the joint region between the malleus and incus at E15.5 (arrow). At P0 (D, F) Meckel's cartilage is still fused to the malleus (arrow). At P3 (E, F), the malleus has separated from Meckel's cartilage (arrow). M, malleus; I, incus; S, stapes; ml, Meckel's cartilage. (A-C taken from Amin & Tucker, 2006, Copyright© 2006 Wiley-Liss, Inc; D-E taken from Anthwal et al, 2013, © 2012 The Authors. Journal of Anatomy © 2012 Anatomical Society).

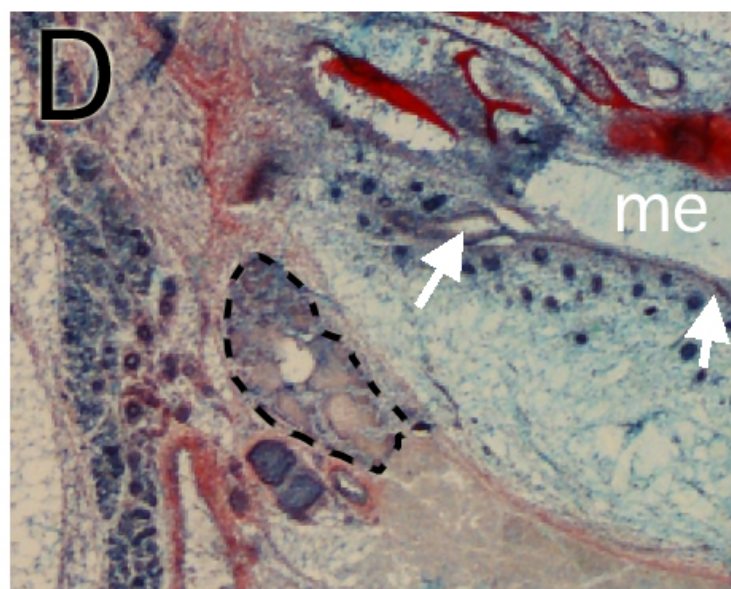
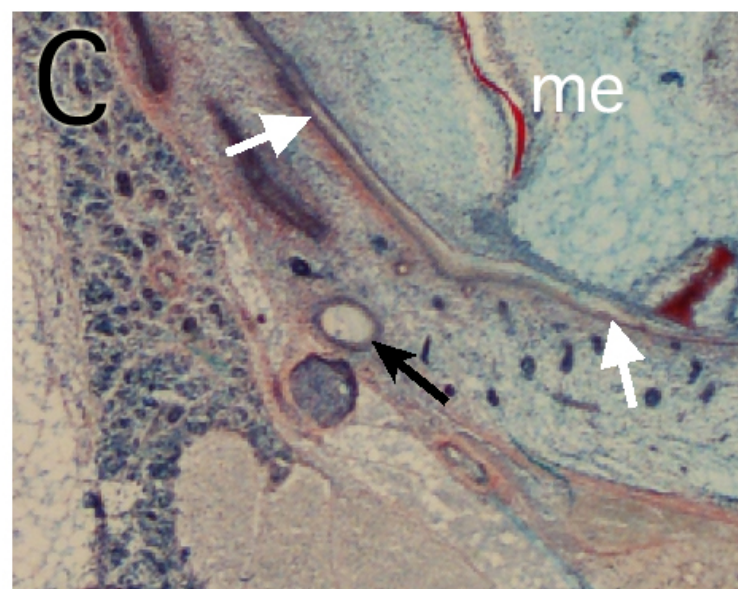
Figure 7. The embryonic origin of the stapes. A) Alcian blue stained stapes at P6, showing it is completely cartilage at this stage. B) X-Gal stained stapes from a P10 *Wnt1cre/R26R* (Danielian et al, 1998; Soriano, 1999) mouse showing part of the footplate as unstained (arrow). C) X-Gal stained stapes from a P10 *Mesp1cre/R26R* (Saga et al, 1999; Soriano, 1999) mouse showing part of the footplate as stained (arrow). D) Schematic illustrating the stapes in the oval window and the orientation of sectioning of E and F. E) Frontal section through a X-Gal stained stapes from a *Wnt1cre/R26R* mouse showing the stained and unstained regions of the footplate and the unstained annular ligament (arrow heads). F) Frontal section through a X-Gal stained stapes from a *Mesp1cre/R26R* mouse showing the stained and unstained regions of the footplate and the stained annular ligament (arrow heads). G) Schematic illustrating the embryonic origin of the stapes in the oval window. Most of the stapes is of neural crest origin (green), while the outer part of the stapedial footplate, the annular ligament (arrow heads) and the otic capsule surrounding the oval window is of mesodermal origin. Sh- head of stapes; c, crus of stapes; fp, footplate of stapes; m, mesodermal footplate of stapes; oc, otic capsule. (A-F taken from Thompson et al, 2012).

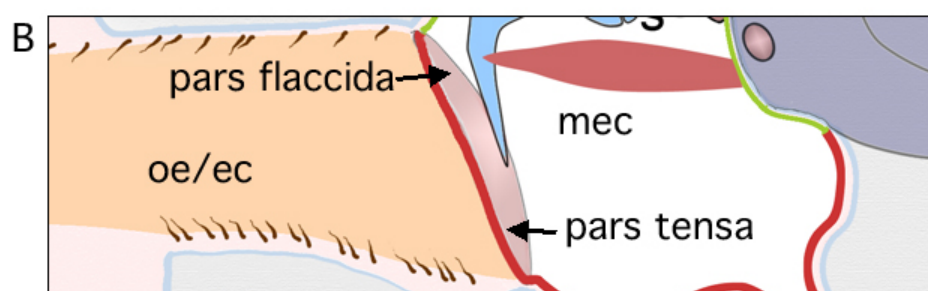
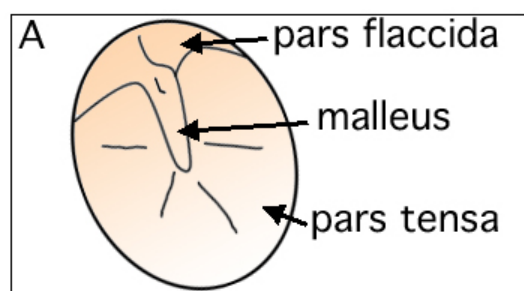
Figure 8. A schematic illustrating the cavitation of the mouse middle ear. A) Representing ~E15.5. The first pharyngeal pouch extends towards the developing middle ear structures. B) Representing ~E17.5 where the tubotympanic recess ruptures and mesenchyme enters the distal end of the cavity. C) Representing ~P6, the structures of the middle ear cavity have grown and developed, the ruptured endoderm is situated towards the edge of the future cavity and the cavity is filled with mesenchyme. D) Representing ~P11, the middle ear mesenchyme is regressing away from the future tympanic membrane. E) Representing ~P13, the middle ear cavity is clear upto the level of the ossicles in the attic region of the cavity. F) Representing ~P16, the middle ear cavity is clear of mesenchyme and the neural crest cells have undergone a mesenchyme to epithelial transformation to form a continuous epithelial lining of the cavity.

Figure 9. The Eustachian tube cartilage is of dual origin. Frontal sections of a *Wnt1cre/R26R* (Danielian et al, 1998; Soriano, 1999) P16 mouse stained with X-Gal. The Eustachian tube cartilage (red dotted line), surrounding the dorsal Eustachian tube (black dash lined), is stained blue (arrow) at its dorsal-most tip, as it is neural crest-derived. However, the majority of the cartilage is unstained and therefore of mesodermal origin. A) shows the Eustachian tube (ET) opening into the middle ear cavity (mec) and is more caudal than B). Dorsal is top.









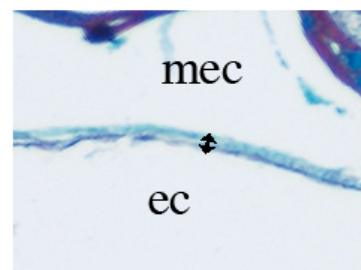
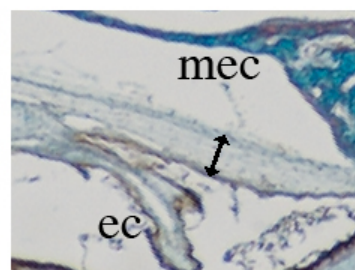
P6

P10

P11

P17

pars flaccida



pars tensa

